

CD4+ T Cells are Exhausted and Show Functional Defects in Chronic Lymphocytic Leukemia

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ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western world. This health problem is caused due to the accumulation of mature B-lymphocytes in the peripheral blood and bone marrow. In the course of cancer, CD4+ T cells become “exhausted” and characterized with poor effector functions and the expression of multiple inhibitory receptors. **Objective:** To investigate the frequency and functional properties of exhausted CD4+ T lymphocytes in patients with CLL. **Methods:** Peripheral blood mononuclear cells were obtained from 25 untreated CLL patients and 15 healthy volunteers. CLL patients were clinically classified according to the Rai staging system. The frequency of CD4+/Tim-3+/PD-1+ cells was obtained by flow cytometry. To evaluate cell proliferation and cytokine production, CD4+ T cells were isolated and stimulated with phytohemagglutinin and PMA/ionomycin. Concentrations of IL-2, IFN- γ , TNF- α , and IL-10 were measured in the culture supernatants of stimulated cells by the ELISA technique. **Results:** The percentage of CD4+/Tim-3+/PD-1+ cells was significantly higher in CLL patients than that of healthy controls. CD4+ T cells from CLL patients showed lower proliferative responses, a lower production of IL-2, IFN- γ , and TNF- α , and a higher production of IL-10, compared to healthy controls. CD4+ T cells from CLL patients in advanced clinical stages showed more exhaustion features than those of early stages. **Conclusion:** Given that the exhaustion phase of T cells can be reversible, targeted blocking of immune inhibitory molecules could be a promising tool to restore the host immune responses against leukemic cells in CLL.

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Keywords: Chronic Lymphoblastic Leukemia, Exhausted CD4+ T Cell, PD-1, Tim-3

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature malignant CD5+ B cells in peripheral blood, bone marrow, and secondary lymphoid organs (1,2). CLL is the most common form of hematologic malignancies of adults in the western world, while it constitutes about 10% of all leukemia in some Asian countries such as China and Japan (3,4). Many patients survive for years without requiring any specific treatment because of an indolent course, while others become symptomatic or develop signs of rapid progression and thus need treatment (1,5). Management of poorly responding patients or those with relapsed or refractory CLL is challenging since they suffer from neutropenia or lymphopenia resulting in immune dysfunction and frequent infections (6).

T cells play a major role in antitumor immunity, as CD4+ helper T cells produce cytokines activating both macrophages and CD8+ cytotoxic T cells (CTLs), while CTLs directly kill the tumor cells (7,8). However, tumor microenvironments could modulate T cell responses leading to disease progression (7). Defects in T cell-mediated antitumor immunity might occur as the result of T cell exhaustion (9), which is developed due to the repeated exposure to the antigens during chronic infections and malignancies (10). Exhausted CD4+ T cells show defects in functional properties such as proliferation and cytokine production (11,12). It has been demonstrated that exhausted T cells play a crucial role in the progression of chronic infections including lymphocytic choriomeningitis virus (LCMV) (13), human immunodeficiency virus (HIV) (14), and hepatitis B virus (HBV) (15), as well as in various malignancies such as acute myeloid leukemia (16) and melanoma (17). T cell exhaustion is associated with up-regulation of a variety of inhibitory immune checkpoint receptors such as T cell immunoglobulin domain and mucin-3 (Tim-3), programmed death-1 (PD-1, CD279), Lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), BY55 (CD160), and 2B4 (CD244) (16,18). Among different identified inhibitory receptors, PD-1 and Tim-3 are two crucial regulatory molecules that have received more attention. Studies in acute myeloid leukemia, melanoma, and LCMV infection have shown that co-expression of PD-1 and Tim-3 on the surface of T cells is associated with a more severe exhaustion features leading to disease progression (16,17,19). PD-Ligand 1 (PD-L1), the main ligand for PD-1, is constitutively expressed on T cells, B cells, macrophages, and dendritic cells (20) and several tumor cells, where this interaction involves in inhibition of the antitumor T cell immunity (21). Despite the established role of the PD-1/PD-L1 pathway in T cell exhaustion, blockade of PD-1 has not been reported to completely restore the T cell function, suggesting the importance of other co-inhibitory receptors such as Tim-3, in T cell exhaustion mechanisms (19,22,23). In our previous study on CLL patients, we showed that CD8+ T cells are exhausted and show functional defects (24). We have also demonstrated the up-regulation of Gal-9 and PD-L1 immune checkpoint molecules in CLL patients as the main ligands of Tim-3 and PD-1, respectively (25). Therefore, we hypothesized that in CLL patients, CD4+ T cells co-expressing PD-1 and Tim-3 are exhausted and have defects in proliferation and cytokine production. Furthermore, the correlation of exhausted CD4+ T cells with disease severity of CLL patients was explored in this survey. A more thorough understanding of the phenotype and function of exhausted CD4+ T cells in CLL may reveal potential therapeutic targets leading to the restoration of CD4+ T cells function and improving the antitumor immunotherapy strategies.

MATERIALS AND METHODS

Study Subjects. In this research, 25 untreated CLL patients (13 males and 12 females) with the age range of 48-84 years (mean age: 62 years) attending the Hematology and Oncology Clinic of Imam Khomeini Hospital affiliated to Mazandaran University of Medical Sciences were enrolled. CLL was diagnosed based on the clinical evaluation, blood cell count, cell morphology, and immunophenotyping analysis according to the World Health Organization (WHO) criteria (1). Disease staging was defined based on the Rai staging system and National Cancer Institute Working Group (NCIWG) criteria (26). Accordingly, patients were classified into early clinical stages (Rai stage 0 and I, n=16) and advanced clinical stages (Rai stage II, III, and IV, n=9) (Table 1). Next, 15 healthy volunteers (9 males and 6 females) age- and sex-matched with patients with the age range of 35-77 years (mean age: 58.2 years) were recruited. Heparinized peripheral blood samples were taken from each study subject after obtaining written informed consents according to the Helsinki Declaration and Ethical Committee of Mazandaran University of Medical Sciences.

Table 1. Major clinical and laboratory characteristics of CLL patients.

No	Age (year)	Sex	WBC×10 ³ / mm ³	Lymph (%)	PLT×10 ³ / mm ³	Hb (g/dl)	Rai Stage	Organomegaly
1	59	M	39.6	79	178	15.0	0	-
2	52	F	63.4	82	38	11.5	II	LAP, SPM
3	49	M	43.3	90	128	10.5	III	LAP, SPM
4	70	M	40.0	90	246	14.1	0	-
5	50	F	107.0	82	62	6.0	IV	LAP, SPM
6	48	F	16.0	74	155	12.2	0	-
7	78	M	26.3	-	199	10.9	0	-
8	50	M	13.4	60	236	11.8	0	-
9	56	M	20.7	56	174	14.5	0	-
10	59	M	29.7	84	124	13.8	II	LAP, SPM
11	73	M	34.9	95	166	12.3	0	-
12	68	F	43.2	82	269	10.8	I	LAP
13	55	F	40.3	93	105	11.2	II	LAP
14	67	M	32.3	87	185	13.9	0	-
15	84	M	18.6	80	307	10.0	0	-
16	67	M	34.8	87	171	12.6	I	LAP
17	72	F	47.2	62	206	10.6	0	-
18	55	M	26.7	76	157	15.3	I	LAP
19	56	M	32.3	76	76	15.6	II	LAP, SPM
20	74	F	41.5	-	191	14.0	0	-
21	64	F	26.9	78	79	12.8	II	LAP
22	81	F	50.6	89	113	11.3	II	LAP
23	50	F	20.0	74	365	11.4	0	-
24	60	F	112.0	94	178	8.7	III	LAP, SPM
25	59	F	33.6	82	240	13.9	I	-

Abbreviation: CLL: chronic lymphocytic leukemia, M: male, F: female, LAP: lymphadenopathy, SPM: splenomegaly, WBC: white blood cell count, Lym: lymphocytes percent in peripheral blood, Hb: hemoglobin and PLT: platelet count.

Isolation of Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood using Ficoll-Histopaque (Biosera, Nuaille, France) density-gradient centrifugation. PBMCs were washed twice with RPMI-1640 culture medium (Biosera, Nuaille, France) and resuspended in the same medium supplemented with 10% (v/v) fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 µg/ml) (Biosera, Nuaille, France). The viability of isolated cells was more than 95% as assessed by trypan blue staining viability method.

Flow cytometric Analysis. PBMCs were stained with flouochrome-conjugated monoclonal antibodies (mAbs) against human antigens: CD4-FITC (Clone SK-3, 5 µL (0.06 µg)/reaction, eBioscience, San Diego, CA, USA), Tim 3-PE (Clone F38-2E2, 5 µL (0.125 µg)/reaction, eBioscience), and PD1-PerCP/Cy5.5 (Clone EH12.2H7, 5 µL (0.125 µg)/reaction, Biolegend, San Diego, CA, USA) together with related isotype-matched controls including FITC-mouse IgG1κ (eBioscience), PE-mouse IgG1K (eBioscience), and PerCP/Cy5.5-mouse IgG1 k (Biolegend). After washing PBMCs with washing buffer (PBS 0.15 M pH: 7.4 with 0.5% BSA), 1×10^6 cells were resuspended in 100 µl of washing buffer and incubated with the appropriate amount of specific mAbs for 45 min at 4°C in the dark. Staining cells were then analyzed on a Partec PAS flow cytometer system (PartecGmbH, Munster, Germany) using the FlowMax software. More than 100,000 events were analyzed for each CLL patient by flow cytometry, among which 10000-12000 events were CD4+ T cells. The same number of CD4+ T cells was also analyzed in normal controls to minimize the variations resulting from differences in the percentage of CD4+ T cells.

CD4+ T Cells Isolation by Magnetic-Activated Cell Sorting. Since both monocytes and CD4+ T cells demonstrate CD4 molecules that may cause interfering results, monocytes were depleted from PBMCs by their plastic adherence property (27). Non-adherent cells were then collected and resuspended in MACS buffer (PBS 0.15 M containing 0.5% BSA, and 2 mM EDTA). CD4+ T cells were isolated from peripheral blood lymphocytes using the CD4 Microbead kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Briefly, after removing clamp and debris with a 70 µm pre-separation filter (Miltenyi Biotec), CD4+ T cells were positively selected by labeling with a mAbs against CD4+ cells conjugated to microbeads according to the manufacturer's instructions. The purity of isolated cells was analyzed by dual-color flow cytometry staining using anti-CD4-FITC (Clone SK-3, 5 µL (0.06 µg)/reaction) and anti-CD3-PE (Clone UCHT1, 5 µL (0.06 µg)/reaction). As expected from positive selection, purity of isolated CD4+ T cells was more than 97%.

Cell Culture and Stimulations. To evaluate T cell proliferation and cytokine production, MACS isolated CD4+ T cells (2×10^5 /well) were cultured in 200 µl of RPMI-1640 medium containing penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% (v/v) FCS (Biosera, Nuaille, France) and incubated at 37°C with 5% CO₂. For proliferation assay, the cells were stimulated with 2 µg/ml of Phytohemagglutinin (PHA) (Sigma-Aldrich, Missouri, USA) for 72 h while for cytokine production the cells were stimulated with PMA/ionomycin cocktail (final concentration of 0.081 µM PMA and 1.34 µM ionomycin, eBioscience) for 6 h.

CD4+ T Cells Proliferation Assay. Following stimulation of CD4+ T cells with PHA, proliferation response was determined using MTT Assay (Sigma-Aldrich, Missouri, USA). Briefly, CD4+ T cells were cultured in 96-well flat-bottom plates at 37°C with 5% CO₂ for 72 h. After that 20 µl of MTT reagent (0.5 mg/ml) was added to each well and cells were incubated for 4 h at 37°C until the purple precipitate was visible. Next,

plates were centrifuged at 300×g for 10 min and then the supernatants were removed and 150 µl of DMSO (Biosera, Nuaille, France) was added as a solvent. Microplates were agitated for 20 min at room temperature to dissolve MTT crystals. The absorbance was recorded at 570 nm on a Multi-scan plate reader (Synergy H1 BioTek, Winooski, USA). Data were expressed as Stimulation Index (SI), which was calculated by dividing the mean ratio of optical density (OD) values obtained from stimulated cells to those of untreated cells.

Cytokine Assay. After stimulation of CD4⁺ T cells with PMA/ionomycin cocktail for 6 h, culture supernatants were collected and used to measure cytokines including IL-2, IL-10, IFN- γ , and TNF- α by Human ELISA Ready Set Go Kits (Sanquin, Amsterdam, The Netherlands).

Statistical Analysis. All statistical analyses were performed using the SPSS 20 statistical package (SPSS, Chicago, USA). All data were expressed as means \pm standard error of mean (SEM). The results were analyzed using Mann-Whitney U test and Spearman correlation test. P-values less than 0.05 were considered as statistically significant.

RESULTS

Percentage and Absolute Count of CD4⁺ T Cells in CLL Patients.

The frequency of CD4⁺ T cells in the peripheral blood was determined in both CLL patients and healthy controls. As expected and shown in Fig. 1A and 1B, the percentage of CD4⁺ T cells in CLL patients was significantly lower than that of healthy controls ($p=0.0004$). However, the absolute number of CD4⁺ T cells was significantly higher in CLL patients than that of healthy controls ($p<0.0001$, Fig. 1C). This difference could be explained by the clonal expansion of tumor-specific CD4⁺ T lymphocytes in CLL patients. Moreover, both percentage and absolute count of CD4⁺ T cells were significantly higher in early clinical stages of CLL patients than those of advanced stages ($p=0.005$ and $p=0.04$, respectively; Fig. 1D and 1E).

Expression of PD-1 and Tim-3 Molecules on CD4⁺ T Cells of CLL Patients.

Since exhausted T cells show a higher expression of inhibitory immune checkpoint molecules, the frequency and the absolute number of PD-1⁺/Tim-3⁺/CD4⁺ T lymphocytes, defined as exhausted CD4⁺ T cells, were measured in CLL patients and healthy controls. Representative flow cytometry plots obtained from a CLL patient and a normal individual are shown in Fig. 2A.

Immunophenotyping analysis showed that the frequency and the absolute number of exhausted CD4⁺ T cells in CLL patients were significantly higher than those of healthy controls ($p=0.0015$ and $p<0.0001$, respectively; Fig. 2B). When an analysis was performed on the expression of either Tim-3 or PD-1 on CD4⁺ T cells, the percentage and the absolute number of Tim-3⁺/CD4⁺ cells and PD-1⁺/CD4⁺ cells were higher in CLL patients compared to those of healthy controls (Figs. 2C and 2D).

CD4⁺ T Lymphocytes from CLL Patients Showed Functional Defects.

The principal characteristics of exhausted CD4⁺ T cells are defined by progressive loss of the T cells function in terms of proliferation and cytokine production. Therefore, we examined the proliferative and cytokine production capacities of MACS isolated CD4⁺

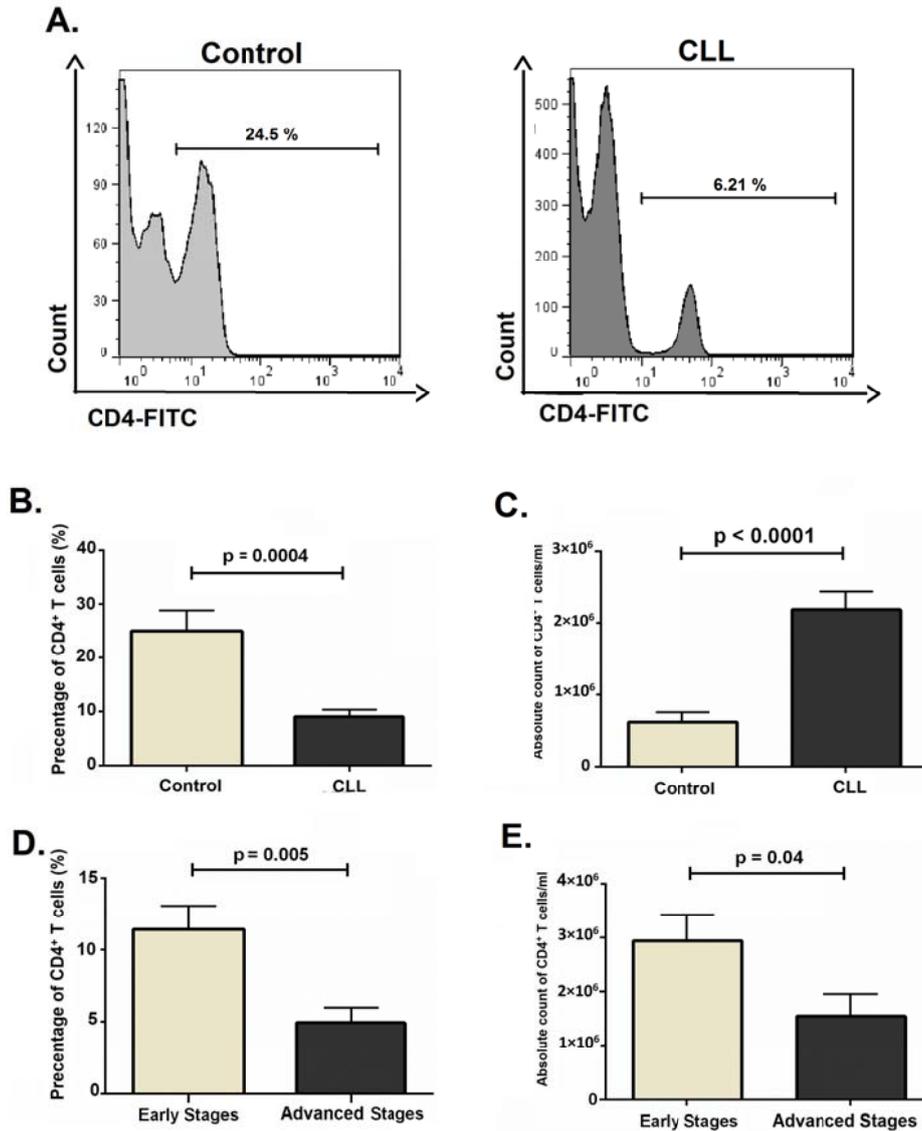


Figure 1. CD4+ T cells frequency in CLL patients and healthy controls. Representative flow cytometric histograms of the CD4+ cells percentage in lymphocyte gate for a CLL patient and healthy control are shown (A). Flow cytometric analysis was used to determine the percentage (B) and absolute number (C) of CD4+ T cells in PBMCs from CLL patients and healthy controls. CD4+ T cells percentage (D) and absolute count (E) are also represented in CLL patients at early and advanced clinical stages. Horizontal bars show the mean values \pm SEM. P-values <0.05 were considered significant.

T cells in response to PHA and PMA/ionomycin, respectively. CD4+ T cells from CLL patients showed lower proliferative responses than those of healthy controls (Fig. 3A), as the stimulation index was markedly lower in CLL patients ($p < 0.0001$, Fig. 3B).

Exhaustion of CD4⁺ T cells in CLL

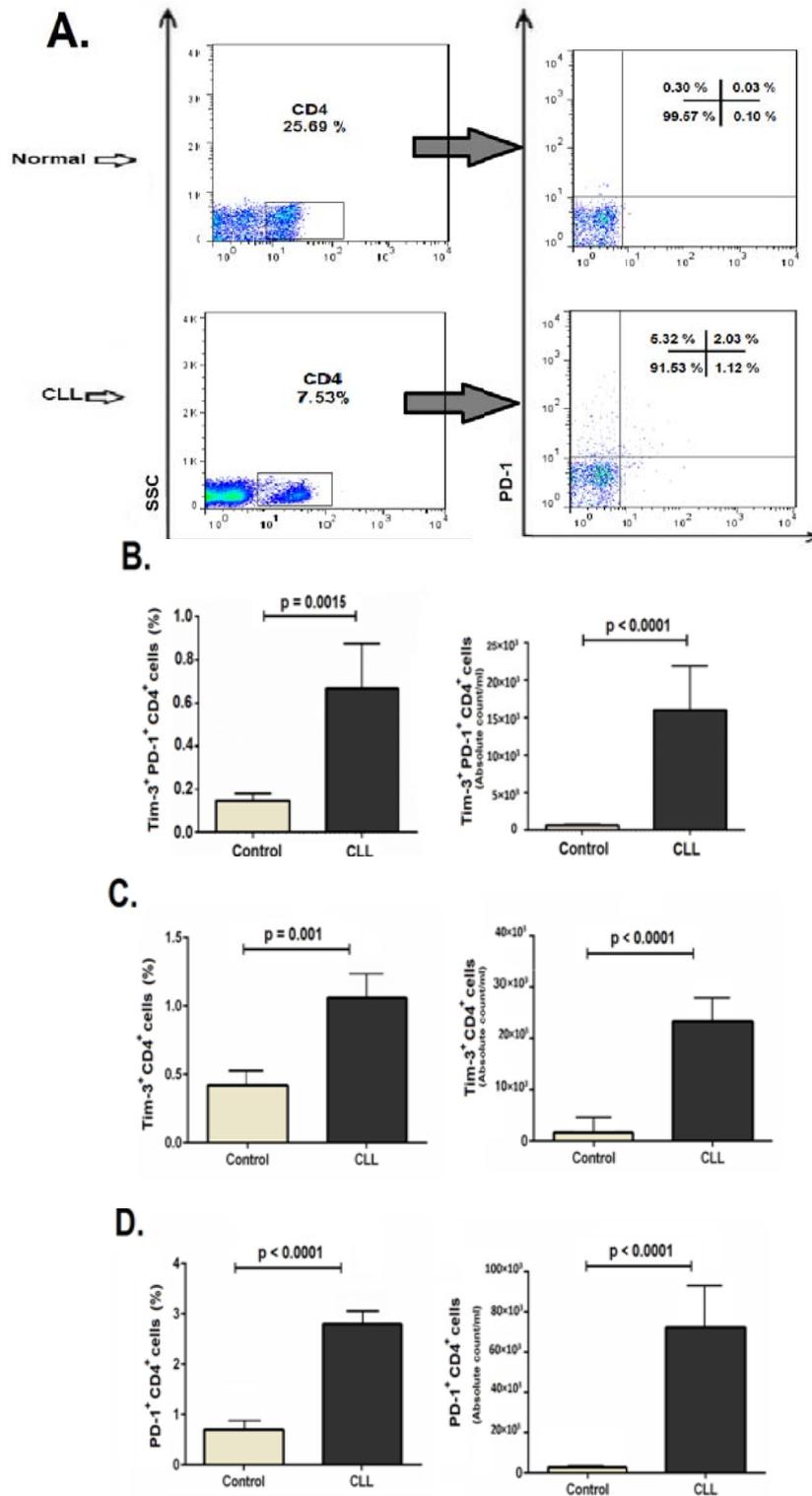


Figure 2. Frequency of exhausted CD4⁺ T lymphocytes in CLL patients and healthy controls. The frequency of Tim-3⁺/PD-1⁺/CD4⁺ T cells in PBMCs, as exhausted CD4⁺ T cells, were determined by three-color flow cytometric analysis. Representative dot-plots indicating the expression of Tim-3 and PD-1 on CD4⁺ T cells in a CLL patient and a healthy control are shown (A). The percentage and absolute number of Tim-3⁺/PD-1⁺/CD4⁺ cells (B), Tim-3⁺/CD4⁺ cells (C), and PD-1⁺/CD4⁺ cells (D) in CLL patients and healthy controls are also represented. Horizontal bars show the mean values ± SEM. P-values <0.05 were considered significant.

Notably, proliferation response of isolated CD4⁺ T cells from CLL patients was inversely correlated with the frequency of exhausted Tim-3⁺/PD-1⁺/CD4⁺ cells, which confirms the low functional properties of these cells. Interestingly, isolated CD4⁺ T lymphocytes from CLL patients produced lower amounts of IL-2, TNF- α , and IFN- γ (Fig. 3C). Concurrently, the production level of IL-10, as a regulatory cytokine, was higher by CD4⁺ T lymphocytes of CLL patients in comparison to healthy controls (Fig. 3C). Moreover, the frequency of exhausted Tim-3⁺/PD-1⁺/CD4⁺ cells was inversely correlated with the production of TNF- α and IL-2 and positively correlated with IL-10 production in CLL patients.

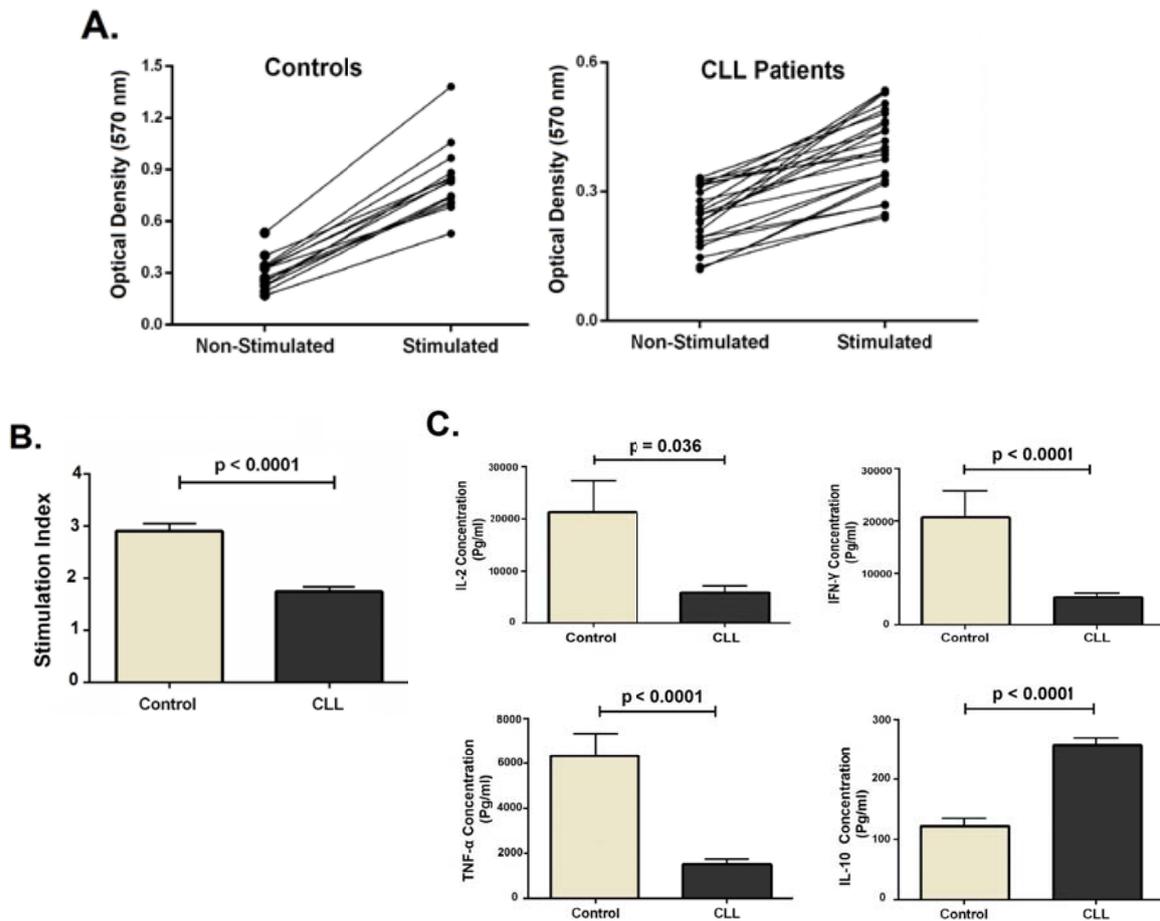


Figure 3. Functional properties of CD4⁺ T cells from CLL patients and healthy controls.

CD4⁺ T cells were isolated from PBLs and then stimulated with PHA and PMA/ionomycin for proliferation response and cytokines production, respectively. Obtained OD values (MTT assay recorded at 570 nm) from untreated and PHA-stimulated cells for CLL patients and healthy controls are represented (A). Stimulation indices (SI) were calculated by dividing the mean ratio of OD values obtained from stimulated cells to those of non-stimulated cells as controls (B). The concentrations of IL-2, IFN- γ , TNF- α and IL-10 in the culture supernatants of stimulated CD4⁺ T cells are shown (C). Horizontal bars show the mean \pm SEM. P-values < 0.05 were considered significant.

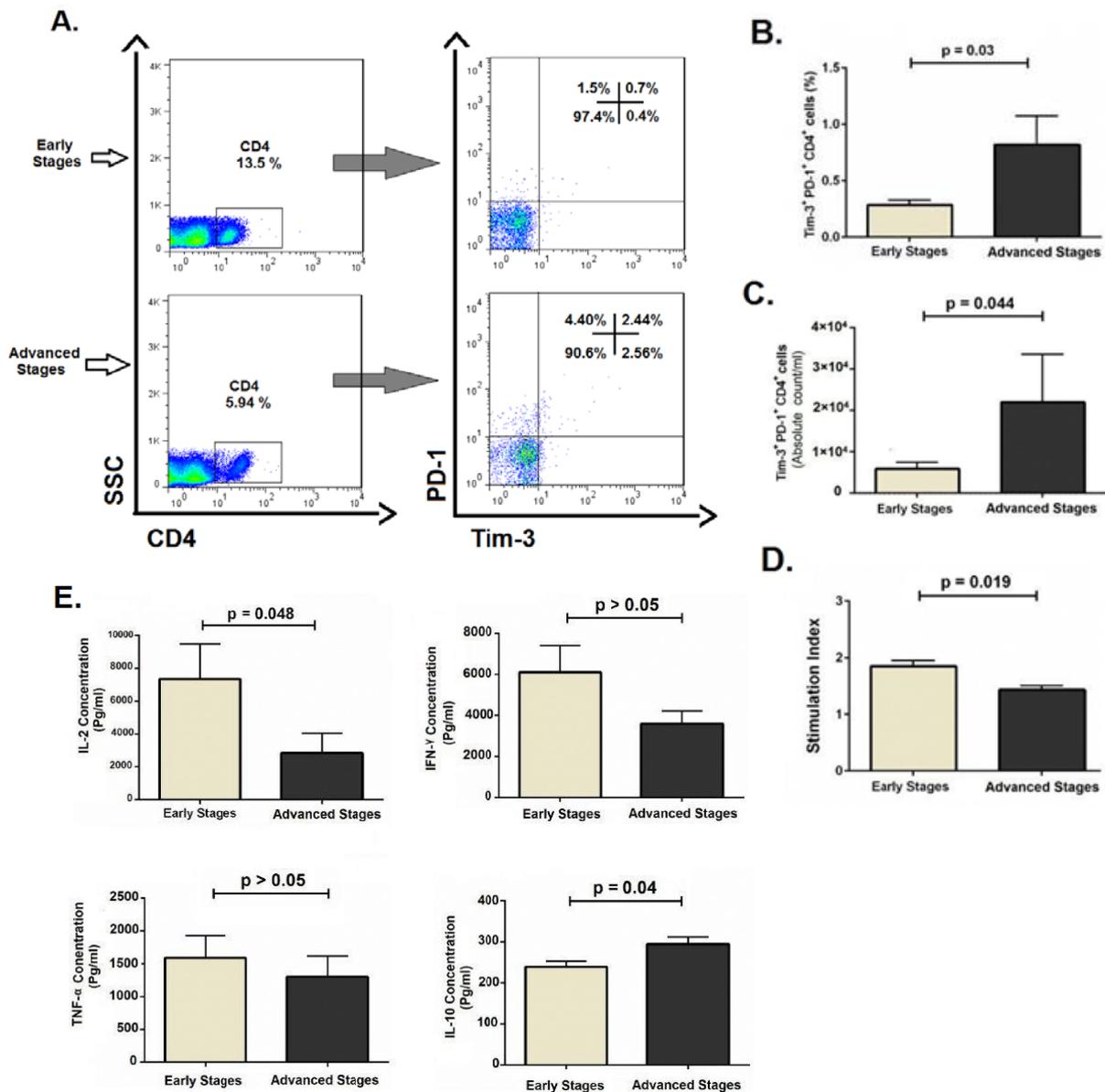


Figure 4. Frequency and functional properties of exhausted CD4⁺ T cells from CLL patients at different clinical stages. The frequency of Tim-3⁺/PD-1⁺/CD4⁺ T cells in PBMCs, as exhausted CD4⁺ T cells, was determined by three-color flow cytometric analysis. Representative dot-plots indicating the expression of Tim-3 and PD-1 on CD4⁺ T cells of two CLL patients at early and advanced clinical stages are shown. To analyze the obtained graphs, CD4⁺ cells were initially gated from lymphocyte population, and then the Tim-3 and PD-1 positive cells of CD4⁺ lymphocytes were determined (A). Percentage (B) and absolute number (C) of Tim-3⁺/PD-1⁺/CD4⁺ cells were compared between CLL patients at early and advanced clinical stages based on Rai staging system. For functional analysis, CD4⁺ T cells were isolated from PBLs and then stimulated with PHA and PMA/ionomycin for proliferation response and cytokines production, respectively. Stimulation indices (SI) were calculated by dividing the mean ratio of OD values obtained from stimulated cells to those of non-stimulated cells as controls (D). The concentrations of IL-2, IFN-γ, TNF-α and IL-10 in the culture supernatants of stimulated CD4⁺ T cells are shown (E). Horizontal bars show the mean values ± SEM. P-values <0.05 were considered significant.

CD4⁺ T Cells from CLL Patients in Advanced Clinical Stages Showed more Features of Exhaustion.

In the next step, the frequency and functional features of exhausted T cells were compared between CLL patients in early clinical stages (Rai stage 0 and I) and advanced clinical stages (Rai stage II, III, and IV). Interestingly, both frequency and the absolute number of Tim-3⁺/PD-1⁺/CD4⁺ cells were significantly higher in advanced clinical stages of CLL patients compared to early stages ($p=0.03$ and $p=0.044$, respectively, Fig. 4A, 4B, and 4C). Isolated CD4⁺ T cells from CLL patients in advanced clinical stages showed more functional defects than those of early stages in terms of proliferation response and production of IL-2, TNF- α , IFN- γ , and IL-10 (Fig. 4D and 4E). However, the results were statistically significant for proliferation response and production of IL-2 and IL-10.

DISCUSSION

T cell exhaustion is a hypo-responsive state of T cells due to the repeated antigen stimulation in chronic infections and cancers. Exhausted T cells show progressive loss of T cell functions in terms of proliferation, cytokine production, and cytotoxicity (18). Expression of inhibitory receptors such as PD-1 and Tim-3 is considered as a hallmark of T cell exhaustion (18,19). Regarding CLL, some studies have evidenced T cell dysfunction with impaired formation of immunological synapse and changes in gene expression pattern (28-30). In this study, we demonstrated the expression level of Tim-3 and PD-1 immune checkpoint molecules on CD4⁺ T cells from CLL patients as well as their association with T cells dysfunction. Our results showed an up-regulation of both Tim-3 and PD-1 on CD4⁺ T cells of CLL patients compared to healthy controls. More interestingly, the frequency of CD4⁺ T cells co-expressing Tim-3 and PD-1 was higher in CLL patients compared to healthy controls. These results are in line with the previous studies in solid (31) and hematopoietic tumors (16), as well as in chronic viral infections (19,32). Regarding functional features of CD4⁺ T cells, we found that CD4⁺ T cells from CLL patients showed impairments in proliferation and cytokine production including IL-2, TNF- α and IFN- γ compared to healthy controls. Moreover, these functional defects were inversely correlated with higher expression of Tim-3 and PD-1 inhibitory receptors on CD4⁺ T cells. Our findings are similar to observations in AML (16), colorectal cancer (31), and chronic viral infections (19). During chronic viral infections, decreasing in production of these cytokines in exhausted CD4⁺T cells was associated with a lack of sufficient CD4⁺ T cell help and an impaired virus-specific CD8⁺ T cell responses (33,34). Contradicting results have also been reported by Riches *et al.* as they showed that although T cells from CLL patients have defects in proliferation and cytotoxicity, they produce increased levels of TNF- α and IFN- γ (9). In this regard, Schnorfeil *et al.* indicated that CD4⁺ and CD8⁺ T cells from AML patients were functionally intact in terms of proliferation and cytokine production, despite increased expression of inhibitory molecules (35). However, recent studies reported that during chronic viral infections, up-regulation of Tim-3 and PD-1 is associated with an increase in IL-10 production (19) and Tim-3 blockade in this infection induces CD4⁺ T cell proliferation and IFN- γ production and decreases the secretion of IL-10, leading to improvements in antiviral immunity (32). Consistent with these results, we observed that CD4⁺ T cells from CLL patients secrete a higher level of IL-10, which is correlated

with the frequency of Tim-3 and PD-1 co-expressing CD4⁺ T cells demonstrating the regulatory features of these cells.

Based on the results of our study, CLL patients with advanced clinical stages showed higher percentages of Tim-3⁺/PD-1⁺/CD4⁺ cells compared to those in early stages of the disease. Therefore, more functional defects in CD4⁺ T cells, in terms of proliferation and cytokine production, are associated with more severe clinical stages of CLL patients. Several lines of evidence indicate that co-expression of Tim-3 and PD-1 is associated with a lower T cell function. Besides, co-blockade of these inhibitory pathways appear to more intensely restore T cell function and inhibit tumor progression (19,22). In this connection, previous studies on chronic infections (34,36) and melanoma (37) have indicated that the restoration of exhausted CD4⁺ T cells could reverse the functional properties of exhausted CD8⁺ T cells and control the disease progression (38). Taken together, it can be assumed that progressive dysfunction of T cells in CLL leads to the substantial impairments in protective immune responses and more progression in disease severity. As previously hypothesized in cancer immunoediting theory, tumor microenvironments suppress cell-mediated immunity using several inhibitory mechanisms to evade immune response mechanisms, and thus targeting these inhibitory pathways can be a useful therapeutic approach (39). To advocate our results, recent studies in tumor immunotherapy using a blocking antibody against inhibitory pathways have demonstrated that in cases with resistance to anti-PD-1 therapy, co-blockade of Tim-3 as an alternative immune checkpoint receptor can improve the anti-PD-1 therapy (23,41). We have also determined the percentage and absolute count of CD4⁺ T cells in the peripheral blood of CLL patients and healthy controls. As expected, the percentage of peripheral blood CD4⁺ T cells was lower in CLL patients than that of healthy controls. Additionally, CLL patients with advanced clinical stages had a lower percentage of CD4⁺ T cells than that of early stages. However, the absolute count of CD4⁺ T cells was higher in CLL patients than in controls, indicating the proliferation and clonal expansion of CD4⁺ T cells responding to the tumor antigens. A former study by D'Arena *et al.* also showed that the absolute number of CD4⁺ T cells was higher in CLL patients than that of healthy controls (40). In conclusion, we reported an elevation in the frequency of CD4⁺ T cells co-expressing Tim-3 and PD-1 in CLL patients. We found that co-expression of Tim-3 and PD-1 on CD4⁺ T cells is associated with the functional defect of CD4⁺ T cells and disease severity in CLL patients. Our findings provide a basis for improving current immunotherapy via blockade of Tim-3 and PD-1 pathways in CLL patients. It remains to examine whether the blockade of these inhibitory receptors could restore functional capacities of exhausted T cells in CLL patients.

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